The Enzymic Synthesis of Amino Acyl Derivatives of Ribonucleic Acid*

IV. THE FORMATION OF THE 3'-HYDROXYL TERMINAL TRINUCLEOTIDE SEQUENCE OF AMINO ACID-ACCEPTOR RIBONUCLEIC ACID

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The previous papers (1–3) and reports from numerous laboratories (4–9) have demonstrated the enzymic synthesis of amino acyl ribonucleic acid derivatives. Studies by Zachau et al. (10), Hecht et al. (11), and ourselves (3) have established that each amino acid is linked to a specific polynucleotide chain through an ester linkage with either the 2'- or 3'-hydroxyl group of the terminal nucleotide residue. In each instance where it has been investigated, the terminal nucleotide has been identified as adenylic acid (3, 10–12). In the amino acid-acceptor ribonucleic acid from mammalian liver it was reported that the adenylic acid unit is linked to a dinucleotide sequence of two cytidylic acid residues (11).

In an attempt to define the nucleotide sequence required for amino acid acceptor activity, we have investigated methods for partially degrading the RNA chains and the requirements for reconverting such degraded preparations to their native state. Removal of one or a few nucleotides from the 3'-hydroxyl end of the polynucleotide chains with purified venom phosphodiesterase destroys the amino acid acceptor activity (3). Such a partially degraded RNA preparation, but not the native material, is an excellent primer for the incorporation of C^{14}-labeled adenosine phosphate and C^{14}-labeled cytidine phosphate from their respective triphosphates by an enzyme obtained from Escherichia coli. Depending upon the extent of degradation of the RNA, the purified enzyme forms part or all of the terminal trinucleotide sequence ... pCpCpA[1] resulting in the restoration of amino acid acceptor activity. The incorporation of each nucleotide unit is accompanied by the liberation of one equivalent of inorganic pyrophosphate. As might be predicted from this finding, the enzyme catalyzes a net pyrophosphorolysis of the intact amino acid-acceptor RNA yielding adenosine triphosphate and cytidine triphosphate from the terminal trinucleotide.

The studies reported here are in agreement with the findings of Hecht et al. (14). These workers reported that after inactivation of mammalian amino acid-acceptor RNA by preincubation with a "pH 5 fraction" from liver or ascites cell extracts,

the incorporation of AMP and CMP by these same extracts increased the amino acid acceptor activity. The utilization of nucleoside triphosphates as precursors for the incorporation of mononucleotide units into RNA has also been reported from several other laboratories. Canallakis and Herbert (15) have partially purified the enzyme for CMP and AMP incorporation from liver, and Hurwitz, Bresler, and Kaye (16) have reported the incorporation of CMP into thymus RNA by an enzyme from thymus. Studies by Harbers and Heidelberger (17) with liver extracts suggest that not only CMP and AMP are incorporated but also GMP and UMP can be linked to the ends of pre-existing RNA chains. The significance of RNA chains terminated by pUpUpG (17) to amino acid acceptor activity is, however, obscure. In contrast to the above mentioned cases which deal almost exclusively with terminal or subterminal nucleotide incorporation, Chung, Mahler, and Envione (18), Edmonds and Abrams (19), Weiss and Gladstone (20), and Stevens (21) have reported the incorporation of mononucleotide units into the internal portion of RNA. The incorporation of AMP reported by Edwards and Abrams (19) does not require the presence of other nucleoside triphosphates, whereas the other authors mentioned above demonstrated a striking stimulation of nucleotide incorporation by the other three naturally occurring nucleoside triphosphates. More recently, Hurwitz, Bresler, and Diringer (22) and Weiss (23) have reported what appears to be a deoxyribonucleic acid-dependent incorporation of ribonucleotides.

EXPERIMENTAL PROCEDURE

Materials

Preparation of Substrates—ATP-8-C^{14} was purchased from Schwarz Laboratories and was judged to be 98% isotopically pure by paper electrophoresis. The ATP-C^{14} had a specific activity of 6.5×10^5 c.p.m. per μmole.

Uniformly labeled CTP-C^{14} was prepared as follows. Uniformly labeled RNA-C^{14} was isolated from Chromatium, strain D, grown with $C^{14}O_2$ as sole source of carbon. The RNA-C^{14} was quantitatively converted to 5'-mononucleotides with purified snake venom phosphodiesterase (24) and the CMP-C^{14}, after separation from the other mononucleotides by chromatography on a Dowex 1-formate column, was converted to CTP with a specific CMP kinase (25) as follows: 1.25 μmoles of CMP-C^{14} (1.02×10^8 c.p.m. per μmole) were incubated for 3 hours at 37°

[1] This designation refers to the convention used by Markham and Smith (13) for adenylyl 5' → 3' cytidylyl 5' → 3' cytidylyl 5' → 3' RNA.

in 5.8 ml with 260 μmoles of Tris buffer (pH 7.4), 30 μmoles of MgCl₂, 30 μmoles of potassium phosphoenol pyruvate, 50 μmoles of KCl, 0.8 μmole of ATP, 1.5 mg of pyruvate kinase (9 μmoles of ATP formed per minute), and 18 units of CMP kinase (1.8 μmoles of CTP formed per hour). After being heated at 100° for 3 minutes, the mixture was adsorbed to a column of Dowex 1-formate, 2% cross-linked (5 × 0.8 cm). The column was eluted sequentially with 0.1 M formic acid, 0.4 M ammonium formate (pH 4.7), and 0.8 M ammonium formate (pH 4.7). This eluted CMP, CDP, and CTP, respectively. The CTP fraction was adsorbed to a second Dowex-formate column, eluted with 6 M formic acid containing 0.3 M ammonium formate, and concentrated by lyophilization. Paper electrophoresis showed that 98% of the radioactivity was associated with CTP, the remainder being CDP. The over-all yield based on the initial amount of CMP was approximately 65%.

ATP³², equally labeled in the two terminal phosphates, was prepared by the leucine-dependent exchange of ATP and PP$_i$³² in the presence of leucyl RNA synthetase (1). The PP$_i$³² was made and isolated as previously described (26).

CTP³², labeled in the terminal pyrophosphoryl group, was obtained by phosphorylating CMP with P³²-acetyl phosphate in the presence of ADP, aceto kinase, and CMP kinase. The incubation and subsequent separation of the CTP³² were performed as follows: 3.8 μmoles of CMP, 3.8 μmoles of ADP, 20 μmoles of P³²-acetylphosphate (1.65 × 10⁸ c.p.m. per μmole), 400 μmoles of Tris buffer (pH 7.5), 5 μmoles of 2-mercaptoethanol, 40 μmoles of MgCl₂, 1 unit of acetokinase (1 μmole of acetylphosphate formed per minute), and 42 units of CMP kinase were incubated in a volume of 7 ml for 2 hours at 37° and heated at 100° for 2 minutes. The mixture was passed through a column of Dowex 1-chloride, 2% cross-linked (4 × 0.8 cm). Elution with 0.01 N HCl-0.05 M KCl gave CMP, CDP³², and ADP in the first 8 bed resin volumes, and CTP³² was eluted between the 16th and 32nd resin bed volume. ATP³² was not eluted until the effluent was changed to 0.01 N HCl-0.2 M KCl. The yield of CTP³² was 72% based on the input of CMP.

The nucleoside mono-, di-, and triphosphates were purchased from the Sigma Chemical Company; DL-leucine-1-C¹⁴ (7.2 × 10⁶ c.p.m. per μmole), DL-valine-1-C¹⁴ (5.5 × 10⁶ c.p.m. per μmole) and the cyclohexylamine salt of phosphoenolpyruvate were obtained from the California Corporation for Biochemical Research. Uniformly labeled dATP-C¹⁴ (2.4 × 10⁶ c.p.m. per μmole) and uniformly labeled dCTP-C¹⁴ (1.2 × 10⁶ c.p.m. per μmole) were prepared as previously described (25, 27).

Amino acid-acceptor RNA was isolated from *E. coli* by the method of Ofengand *et al.* (28). The concentration of RNA is expressed throughout this paper in micromoles of RNA nucleotide, and an optical density of 10.0 at 260 mμ at pH 12 has been used as the equivalent of 1 μmole of nucleotide.

For the assay of the enzymatic rate of AMP-C¹⁴ and CMP-C¹⁴ incorporation, two types of RNA were used as nucleotide acceptor. These differed only with respect to the extent of the phosphodiesterase digestion of the RNA. The procedure used for the preparation of these substrates was as follows: 35 μmoles of RNA nucleotide was incubated with 80 to 160 units of purified venom phosphodiesterase (1 unit liberates 1 μmole of 5'-mononucleotide per hour when measured on a DNase-limit digest (27)), 300 μmoles of glycine buffer (pH 8.5), and 40 μmoles of MgCl₂ in a volume of 3.5 ml at 37°. The reaction was stopped after 15 minutes (for use as the AMP acceptor) or after 60 min-

utes (for the CMP acceptor) by heating at 100° for 90 seconds. A 15-ml solution containing 0.5 M NaCl and 67% ethanol was added, and after 1 hour at 0°, the mixture was centrifuged, the precipitate was dissolved in 10 ml of water, and the solution was dialyzed overnight against 0.02 M Tris buffer, pH 7.4, containing 0.001 M Versenate, followed by dialysis for 2 hours against water. Any insoluble material was removed by centrifugation and discarded. For other preparations of diesterase-digested RNA, only the amount of venom diesterase or the time of incubation was varied.

Purified snake venom phosphodiesterase was prepared from lyophilized venom of *Crotalus adamanteus* (Ross Allen Reptile Farms) by the procedure of Koerner and Sinsheimer (29). It was free of phosphomonoesterase activity when tested with P³²-5'-mononucleotides. Acetokinase was made following the procedure of Rose *et al.* (30), and pyruvate kinase was purchased from the Sigma Chemical Company. The leucyl- and valyl RNA synthetases were prepared as previously described (1).

DEAE-cellulose (California Corporation for Biochemical Research) was washed with water to remove fine particles, and it was suspended in 0.5 N NaOH for 20 minutes and then washed with water until neutral. *E. coli* cells were grown and harvested as already described (31).

Methods

Protein was determined by the method of Lowry *et al.* (32) and P$_i$ according to Fiske and SubbaRow (33). PP$_i$ was measured as P$_i$ after hydrolysis by 1 N acid for 10 minutes at 100°. The formation of leucyl- and valyl RNA was determined as previously described (1).

Measurement of Enzyme Activity for AMP and CMP Incorporation—The enzyme assay measured the rate of conversion of the radioactivity from ATP-C¹⁴ and CTP-C¹⁴ to an acid-insoluble form. The reaction mixture contained, in a volume of 0.5 ml, 20 μmoles of potassium phosphate buffer (pH 7.5), 5 μmoles of MgCl₂, 5 μmoles of 2-mercaptoethanol, 0.8 to 1.0 μmole of diesterase-treated RNA, 0.1 μmole of ATP-C¹⁴ (6.5 × 10⁵ c.p.m. per μmole) or 0.03 μmole of CTP-C¹⁴ (7.3 to 10 × 10⁵ c.p.m. per μmole), 5 to 30 × 10⁻⁴ units of enzyme, and with fractions other than the most purified one, 1 μmole of phosphoenol pyruvate and 50 μg of pyruvate kinase. Where AMP-C¹⁴ incorporation was being measured, 0.1 μmole of unlabeled CTP was also added. After 20 minutes at 37°, the reaction was terminated by the addition of 0.05 ml of cold 3.5 N perchloric acid and 0.8 mg of bovine serum albumin as carrier. The precipitate was centrifuged, washed twice with 3 ml of 0.35 N perchloric acid, dissolved in 1 ml of 1.5 N NH₄OH, and 0.5 ml aliquots were dried on planchets for counting in a windowless gas flow counter. Correction was made for self-absorption and the amount of nucleotide incorporated was calculated after subtraction of the control (the same mixture with no enzyme or no RNA) on the basis of the specific activity of the substrate. Under the above conditions, the rate of AMP-C¹⁴ and CMP-C¹⁴ incorporation was linear for at least 40 minutes. One unit of activity was defined as the incorporation of 1 μmole of nucleotide in 60 minutes. Fig. 1 shows that the rate of nucleotide incorporation was proportional to the amount of enzyme up to about 0.004 unit of enzyme activity (1.3 mμmoles per 20 minutes); above this, the rate was not proportional to enzyme concentration.
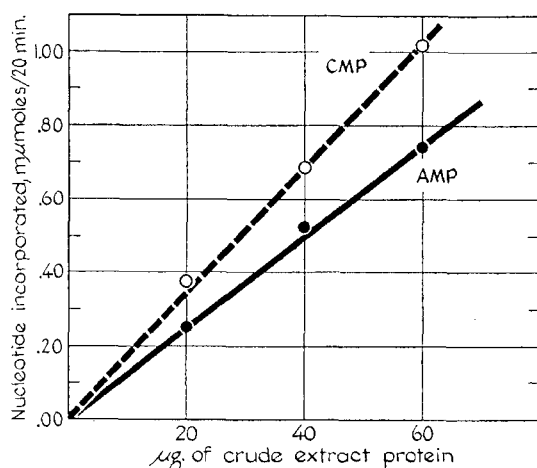
FIG. 1. The rate of AMP-C^{14} and CMP-C^{14} incorporation into RNA as a function of enzyme concentration.

RESULTS

Preparation of Enzyme—Extracts of *E. coli* in 0.05 M Tris buffer (pH 7.4), containing 0.01 M MgCl$_2$, were prepared with glass beads in a Waring Blendor as previously described (31). The crude extract was spun in a Spinco model L centrifuge at 30,000 r.p.m. for 90 minutes and the precipitate discarded. No loss in activity occurs, but a latent RNase, present in the ribosomes (34), is eliminated.

To each 250 ml of stirred supernatant fluid were added 50 ml of 5% streptomycin sulfate, and after 10 minutes the precipitate was centrifuged for 5 minutes at 13,000 × *g* and the precipitate discarded. To the supernatant fluid (292 ml) were added 2.5 ml of 0.3 M reduced glutathione and 106 g of ammonium sulfate, and after 15 minutes the mixture was centrifuged as described above. The precipitate was dissolved in 75 ml of 0.05 M Tris buffer (pH 7.5) containing 0.001 M glutathione and 0.001 M Versenate. The solution was dialyzed against 3 liters of the above buffer for 6 to 8 hours, and enough 0.3 M gluta-

TABLE I

Purification of enzyme incorporating AMP and CMP into amino acid-acceptor RNA

DEAE-fractions A through E represent different regions of the eluted peak of enzyme activity. This was done in this particular experiment to examine the ratio of activity for CMP and AMP incorporation. In the usual preparative procedure, the tubes comprising the peak were pooled and processed as described in the text to yield purified DEAE-fractions.

Fraction	Volume	Total activity		Specific activity		Ratio of activity CMP/AMP
		CMP	AMP	CMP	AMP	
	ml	*μmoles/hour*		*μmoles/hour/mg protein*		
Crude extract	250	154	238	0.030	0.046	0.65
Ammonium sulfate	80	156	222	0.11	0.16	0.69
DEAE-fractions						
A	4.2	6.6	10.8	1.8	3.0	0.60
B	4.2	9.3	14.4	3.2	4.9	0.65
C	4.2	7.8	11.7	3.1	4.8	0.65
D	8.2	14.7	25.5	2.4	4.2	0.57
E	3.8	5.4	9.1	1.7	2.9	0.59

TABLE II

Requirements for incorporation of AMP and CMP into RNA

The assay conditions were described in "Methods." ATP, GTP, or UTP, 100 mμmoles, was used where indicated.

Components	Nucleotide incorporation	
	AMP	CMP
	μmoles/hour/mg protein	
Experiment 1		
Complete system (undigested RNA)	0.28	0.20
Complete system (digested RNA)	2.3	3.6
Complete system minus MgCl$_2$	<0.1	<0.1
Complete system minus RNA	<0.1	<0.1
Complete system minus enzyme	<0.1	<0.1
Experiment 2		
Complete system	2.2	3.6
Minus CTP	0.6	
UTP replacing CTP	0.7	
GTP replacing CTP	0.5	
Complete system plus UTP and GTP	1.9	
Complete system plus ATP		3.2
Complete system plus ATP, GTP, and UTP		3.3

TABLE III

Requirement for CTP and ATP as substrate for CMP and AMP incorporation into amino acid-acceptor RNA

The conditions of the experiment are described under "Methods."

Substrates and additions	Nucleotide incorporation
	μmoles/hour/mg protein
CTP-C^{14}, 0.03 μmole	1.53
CTP-C^{14}, 0.03 μmole + CMP, 0.29 μmole	1.53
CTP-C^{14}, 0.03 μmole + CDP, 0.30 μmole	1.38
CTP-C^{14}, 0.03 μmole + dCTP, 0.34 μmole	1.30
dCTP-C^{14}, 0.04 μmole	<0.05
ATP-C^{14}, 0.1 μmole	1.50
ATP-C^{14}, 0.1 μmole + AMP, 1.3 μmole	1.50
ATP-C^{14}, 0.1 μmole + ADP, 1.3 μmole	1.20
ATP-C^{14}, 0.1 μmole	1.34
ATP-C^{14}, 0.1 μmole + dATP, 1.1 μmole	1.16
dATP-C^{14}, 0.05 μmole	<0.01

thione was then added to the dialyzed enzyme fraction to make a final concentration of 0.01 M (ammonium sulfate fraction).

The ammonium sulfate fraction was adsorbed on a DEAE-cellulose (type 40) column (24 × 2.2 cm), and the enzyme was eluted with a linear gradient of increasing phosphate concentration. The mixing chamber contained 500 ml of 0.005 M potassium phosphate buffer, pH 7.8, with 0.005 M glutathione, and the reservoir contained 500 ml of 0.08 M potassium phosphate buffer, pH 7.8, and 0.005 M glutathione. Fractions of about 15 to 20 ml were collected into tubes containing 0.07 ml of 0.3 M glutathione at a flow rate of about 90 ml per hour. After analysis of the contents of each tube for enzyme activity and protein

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TABLE IV

Equivalence between AMP and CMP incorporation into RNA and PP_i formation

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The reaction mixtures were the same as those described for the standard assay except that 20 μmoles of Tris buffer, pH 7.4, was substituted for the phosphate buffer and 5 μmoles of KF was added to minimize the action of inorganic pyrophosphatase. Parallel mixtures containing 34 mμmoles of ATP-C^{14} (6.5 $\times$ 10^5 c.p.m. per μmole) or 34 mμmoles of AMPP^{32}P^{32} (7.9 $\times$ 10^6 c.p.m. per μmole) were incubated for 2 hours at 37° in the presence or absence of RNA digested with venom diesterase. Essentially identical mixtures were set up with 7.4 mμmoles of either CTP-C^{14} (1 $\times$ 10^6 c.p.m. per μmole) or CMPP^{32}P^{32} (3.4 $\times$ 10^7 c.p.m. per μmole) and incubated as above. AMP-C^{14} and CMP-C^{14} incorporation were measured as already described.

The P^{32}-containing reaction mixtures were analyzed as follows. The reaction was terminated by the addition of 2 ml of 0.35 N cold perchloric acid. Serum albumin, 1.2 mg, and P_i and PP_i, 10 μmoles each, were added as carrier. The precipitate was washed twice with 1.5 ml of the perchloric acid and to the combined wash and supernatant fluids were added 30 mg of acid-washed Norit to adsorb the unreacted nucleoside triphosphates. The mixture was centrifuged, and the Norit was washed twice with 0.01 N HCl. The combined supernatant and wash fluids were adjusted to about pH 8 with NaOH and adsorbed to a Dowex 1-Cl⁻ column. The P_i and PP_i were eluted with 0.01 N HCl-0.05 M KCl, and the radioactivity in each fraction was determined. The P_i and PP_i were identified by their position in the elution pattern and by analysis for P_i and acid-labile P_i. The amount of $PP_i{}^{32}$ found was calculated from the total radioactivity recovered in the PP_i peak and the specific activity of the AMPP^{32}P^{32}.

The residual CMPP^{32}P^{32} was determined by counting the Norit pellet. No attempt was made to analyze for the untreated AMPP^{32}P^{32} inasmuch as the utilization represented only about 10% of the initial ATP.

	Complete	Minus RNA	Δ
	mμmoles		
Experiment 1			
AMP incorporated	5.5	0.0	+5.5
P_i found	1.7	2.4	−0.7
PP_i found	6.3	0.4	+5.9
Experiment 2			
CTP remaining	0.9	7.1	−6.2
CMP incorporated	6.4	0.0	+6.4
P_i found	0.8	1.1	−0.3
PP_i found	6.1	0.0	+6.1

concentration, the fractions most active for the incorporation of the two nucleotides were pooled and concentrated by lyophilization. The position of the peak varied somewhat from one preparation to another, but it was usually obtained after 350 to 450 ml of eluate had been collected. On several occasions, the enzymatic activity was eluted from the column in two overlapping peaks rather than the usual single peak. However, the ratio of the two incorporating activities throughout the twin peaks was within the same limits shown in Table I. The reasons for the activity occasionally appearing in two peaks is unknown.

A summary of the results of a typical preparation are shown in Table I. The constant ratio of the rates of incorporation of AMP and CMP throughout the purification of the enzyme leads us to the tentative conclusion that both AMP and CMP

are incorporated into the amino acid-acceptor RNA by a single enzyme. More definitive evidence must await further purification of the incorporating activity.

Components Required for Incorporating AMP and CMP into Terminal Trinucleotide Portion of Amino Acid-Acceptor RNA— The formation of C^{14}-labeled RNA from ATP-C^{14} or CTP-C^{14} required the presence of enzyme, Mg^{++}, and a preparation of venom diesterase-digested amino acid-acceptor RNA (Table II, Experiment 1). Substitution of the partially degraded RNA by untreated RNA (*i.e.* active as amino acid acceptor) resulted in a decrease in the rate of reaction to about 10% or less. *E. coli* ribosomal RNA (28) was less than 5% as active as the partially digested amino acid-acceptor RNA, and its activity was not changed by an equal amount of digestion with the venom diesterase. In a later portion of this paper, it will be shown that the incorporated AMP and CMP formed a terminal trinucleotide sequence in the RNA.

Although the addition of a nucleoside triphosphate-regenerating system (pyruvate kinase and phosphoenol pyruvate) had no effect on the reaction rate with the purified enzyme fraction, there was a 2-fold increase in the activity with the original extract and ammonium sulfate fractions. Presumably, this reflects the presence of nucleoside triphosphate-degrading enzymes in the cruder fractions.

The incorporation of CMP from CTP was unaffected by the presence of GTP, UTP, or ATP or by a mixture of these compounds (Table II, Experiment 2). By contrast, the rate of AMP incorporation was increased by about 3-fold in the presence of CTP but not by GTP or UTP. It should be pointed out here that the effect of added CTP is a function of the extent of digestion of the acceptor RNA by the diesterase, and in certain preparations of RNA in which both CMP residues have been removed, the incorporation of AMP is essentially completely dependent on the incorporation of CMP.

*Requirement of ATP and CTP as Nucleotide Donors and Identification of PP_i as Product of Reaction—*The substrates for AMP and CMP incorporation into RNA are ATP and CTP, respectively (Table III). The addition of a 10-fold excess of unlabeled nucleoside mono- or diphosphate or the corresponding deoxynucleoside triphosphate does not appreciably dilute the incorporation of AMP-C^{14} from the ATP-C^{14}. Moreover, neither dATP nor dCTP serve as nucleotide donors.

The incorporation of AMP and CMP from their respective triphosphates leads to the formation of PP_i (Table IV). Moreover, PP_i is formed only in the presence of RNA and in amounts equivalent to the amount of AMP or CMP linked to the RNA. It is also seen that the amount of CTP which disappears is equivalent to the CMP incorporation and to the amount of PP_i formation.

*Demonstration that Venom Diesterase-digested RNA Functions Stoichiometrically as AMP and CMP Acceptor—*Under the usual assay conditions, AMP and CMP incorporation ceased after about 60 minutes (Fig. 2a and b). Further addition of ATP or CTP and enzyme had no effect on the amount of nucleotide incorporated. The addition of more RNA resulted in a renewed incorporation of the two nucleotides. In experiments with an excess of enzyme, and in which only the amount of diesterase-treated RNA added to the reaction was varied, there was a proportional increase in the extent of incorporation of the two nucleotides (Fig. 3).

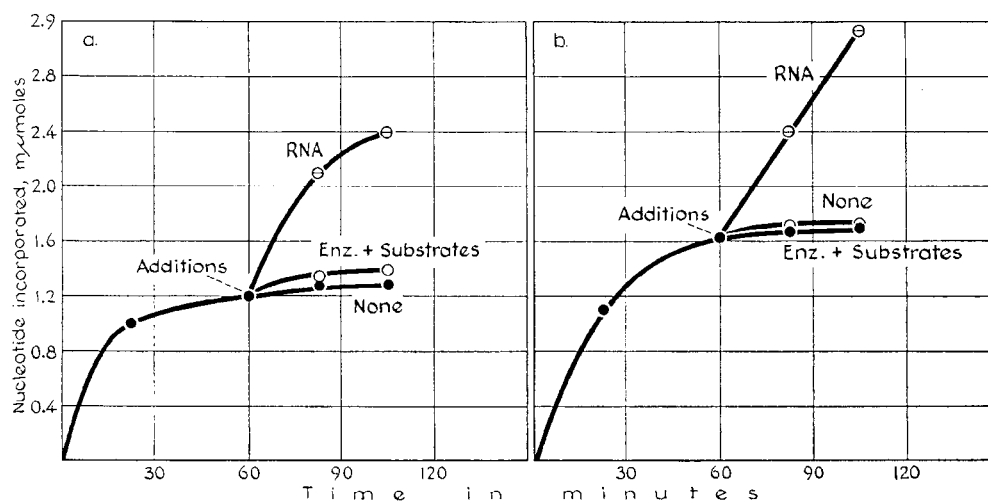*Relation Between Extent of Digestion of Amino Acid-Acceptor*

FIG. 2. The extent of AMP and CMP incorporation as a function of the amount of diesterase-treated RNA. *Curve a*, AMP incorporation; *Curve b*, CMP incorporation. The conditions were those described in "Methods."
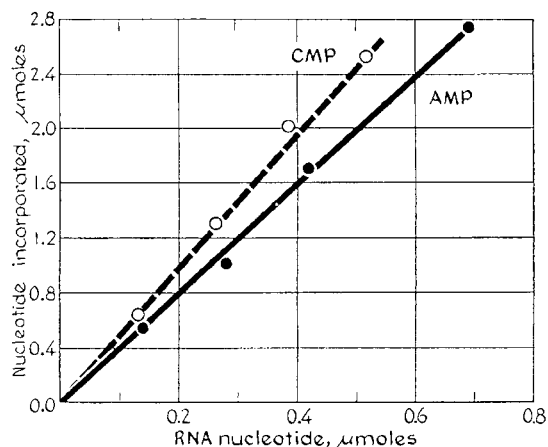

FIG. 3. The effect of the amount of RNA nucleotide added on the extent of CMP and AMP incorporation. The initial reaction mixture was that described in the text. The reaction was allowed to proceed to a limit, and then an additional amount of enzyme (2 μg) and CTP-C^{14} (30 mμmoles) or ATP-C^{14} (100 mμmoles) were added. In a parallel experiment, an amount of RNA nucleotide equivalent to the initial input was added. The reaction was allowed to proceed for an additional 40 minutes and samples were analyzed for nucleotide incorporation as described in "Methods."

RNA and Rate and Extent of Nucleotide Incorporation—Amino acid-acceptor RNA, isolated on the basis of its amino acid acceptor capacity (28), is a poor acceptor of AMP or CMP. Fig. 4a shows the effect of progressive digestion of the RNA by the diesterase on the rate of AMP and CMP incorporation. Note that the rate of AMP incorporation into the RNA, recovered after a short period of diesterase digestion, was greatly increased over the untreated RNA and that this effect diminished with further digestion of the RNA. The rate at which CMP was incorporated into the RNA, on the other hand, increased as the acceptor RNA was degraded progressively by the diesterase. The rate of CMP incorporation began to decline only after more extensive degradation by the diesterase. Fig. 4b presents the effect of progressive diesterase digestion on the *extent* to which AMP and CMP can be linked to the RNA. If AMP incorporation was measured in the absence of CTP, there was first an increase and then a rapid decline in the capacity to

accept AMP. In the presence of CTP, however, the extent of AMP incorporation increased to a considerably higher level. The extent of incorporation of CMP also increased as digestion of the RNA proceeded but there was no dependence on ATP. These data suggest that CMP incorporation must precede AMP incorporation.

The reasons for the decline in the rate of AMP incorporation as the RNA was degraded to a greater extent by the diesterase are not clear. This occurred even though the total amount of AMP which can be incorporated continues to increase (Fig. 4b).

Table V summarizes the effect of varying degrees of venom diesterase-digestion of the amino acid-acceptor RNA on the extent of AMP and CMP incorporation. With each of the conditions so far studied, the ratio of the final amount of CMP to AMP incorporated increases and approaches a value of 2. Even when, after relatively extensive degradation, the amount of incorporation of both nucleotides begins to decrease, the ratio remains near 2. In no case has the incorporation of AMP and CMP been affected by the addition of UTP and GTP. The data indicate, therefore, that the purified enzyme can incorporate both AMP and CMP into the degraded amino acid-acceptor RNA at the AMP to CMP ratio of 1:2.

Determination of Position and Sequence of Incorporated CMP and AMP—Venom diesterase is known (35, 36) to attack preferentially the terminal nucleotide units of polynucleotide chains, liberating 5'-mononucleotides and exposing the 3'-hydroxyl group of the adjacent nucleotide (Fig. 5). Alkaline hydrolysis of a polynucleotide chain terminated by free 2'- and 3'-hydroxyl groups liberates the terminal nucleotide as a nucleoside and nonterminal nucleotides as a mixture of 2'- and 3'-mononucleotides (37). An analysis of the products formed after alkaline hydrolysis of RNA, to which had been added either CMP-C^{14} or AMP-C^{14}, is shown in Table VI. First, note that essentially all of the incorporated AMP-C^{14} is found in a terminal position even though it was incorporated in the presence of CTP. The addition of CMP-C^{14} to a preparation of RNA which could accept on the average 1 CMP residue for each AMP unit was at least 87% terminal. If, however, the incorporation of CMP-C^{14} was followed by the addition of AMP, all of the CMP-C^{14} was converted to a nonterminal position. When CMP-C^{14} was linked to an RNA preparation which could accept approximately 2 CMP residues per AMP, 41% of the CMP-C^{14} was nonterminal
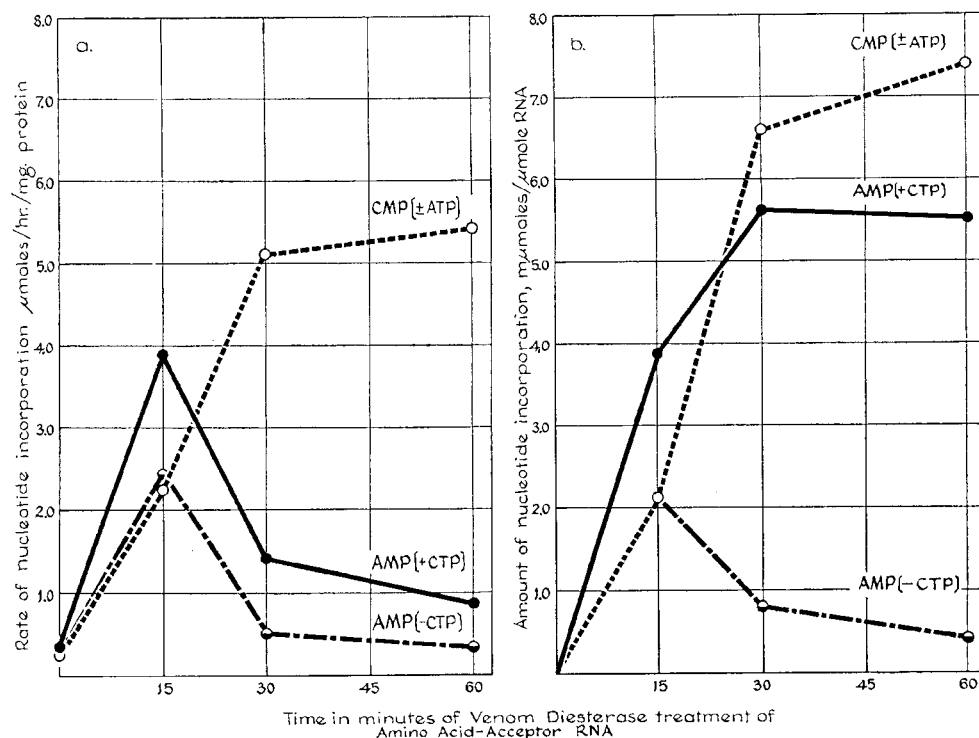
Fig. 4a and b. The effect of venom diesterase digestion of amino acid-acceptor RNA on the rate and extent of AMP and CMP incorporation. The measurements of the rate and extent of nucleotide incorporation into the various RNA preparations were performed as described in "Methods."

TABLE V

Effect of venom diesterase digestion of amino acid-acceptor RNA on extent of AMP and CMP incorporation

The RNA preparations were treated with venom diesterase and isolated as described under "Methods." The amount of AMP and CMP incorporation was measured under conditions where the reaction had reached a limit as described earlier.

Amount of diesterase	Time of digestion	Nucleotide incorporation		Ratio of incorporation CMP/AMP
		CMP	AMP	
units/μmole RNA nucleotide	*min*	*mμmoles/μmole RNA nucleotide*		
0		0.16	0.13	1.23
1.0	10	1.2	3.3	0.36
1.0	20	2.3	3.7	0.62
1.0	40	4.2	4.5	0.93
1.0	90	5.4	5.6	0.97
2.7	5	2.0	3.8	0.53
2.7	30	6.4	4.8	1.33
2.7	60	9.4	5.9	1.59
2.7	120	8.8	4.7	1.87
5.2	30	6.9	3.6	1.92
5.2	60	7.3	3.8	1.92

and the rest terminal. Again, if AMP was subsequently linked to the RNA, all of the CMP was converted to nonterminal residues. These findings establish that the incorporated AMP and CMP compose a terminal trinucleotide sequence, adenylyl 5' → 3' cytidylyl 5' → 3' cytidylyl 5' → 3' RNA.

Pyrophosphorolysis of Amino Acid-Acceptor RNA—When intact amino acid-acceptor RNA was incubated with PP_i^{32} and the nucleotide-incorporating enzyme, the PP_i^{32} was converted to compounds which were adsorbed to Norit (Fig. 6a). This formation of Norit-adsorbable P^{32} was entirely dependent on the presence of the RNA. Moreover, RNA labeled in the terminal trinucleotide with CMP-C^{14} and AMP-C^{14} lost the C^{14} label in the presence of the enzyme and PP_i (Fig. 6b). Experiments are still in progress to determine whether the incomplete pyrophosphorolysis represents a true equilibrium value or whether the reaction becomes limiting for other reasons.

Under the conditions described above, pyrophosphorolysis of intact amino acid-acceptor RNA in the presence of PP_i^{32} resulted in the formation of ATP^{32} and CTP^{32} in approximately equal amounts, but formed no UTP or GTP (Table VII). Moreover, the RNA isolated from the reaction mixture can accept CMP and AMP in the ratio of 1.2. By contrast, there was no PP_i^{32} uptake into nucleoside triphosphates when RNA isolated from the ribosomal particles was used as substrate. When RNA, which has been pretreated with venom diesterase to the point where 1 CMP and 1 AMP have been removed is pyrophosphorolyzed, ATP^{32} and CTP^{32} are also formed but in lesser amounts, and there is about 4 to 5 times more CTP^{32} than ATP^{32}. The ATP^{32} is presumably formed from those RNA chains which still have an intact pCpCpA end group (see later section) and the CTP^{32} is derived from those chains which have either 1 or 2 CMP residues remaining. This finding shows that the enzyme pyrophosphorolyzes the same residues which are removed by the venom diesterase. The above findings establish that the nucleotide-incorporating enzyme catalyzes a net pyrophosphorolysis of amino acid-acceptor RNA and that the products are ATP and CTP.
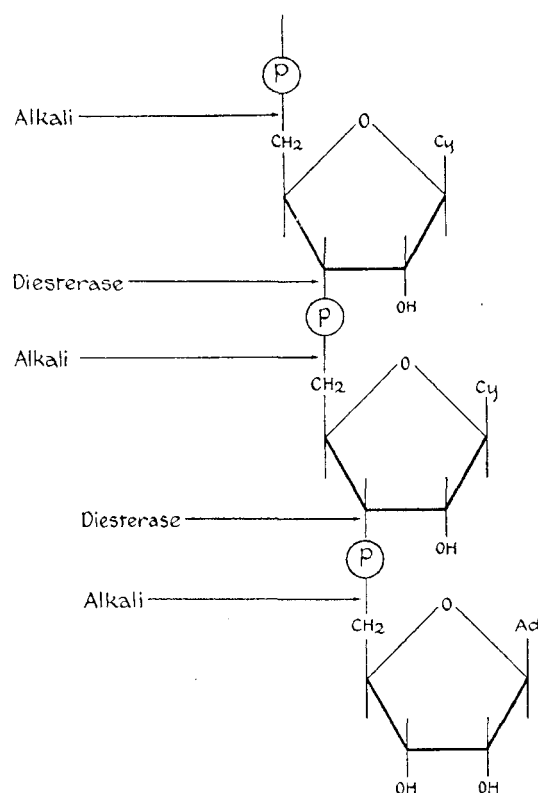
FIG. 5

FIG. 6. The pyrophosphorolysis of amino acid-acceptor RNA
a. The reaction mixtures contained in 0.5 ml, 20 μmoles of Tris buffer (pH 7.5), 3 μmoles of PP_i^{32} (8.7 $\times$ 10^5 c.p.m. per μmole), 0.46 μmole of RNA nucleotide, and 0.02 unit of enzyme. Incubation was carried out at 37°, and the reaction was terminated by the addition of 0.05 ml of 3.5 N perchloric acid followed by 1.2 mg of bovine serum albumin and 1.5 ml of 0.35 N perchloric acid. The precipitate was removed by centrifugation, and it was washed with 2 ml of 0.35 N perchloric acid. To the combined supernatant and wash fluid were added 10 μmoles of unlabeled PP_i as carrier, about 0.3 μmole each of CTP and ATP, and 30 mg of acid-washed Norit. The mixture was centrifuged, and the Norit was washed three times with 0.35 N perchloric acid containing 0.001 M PP_i, suspended in 50% ethanol containing 0.3 M NH_4OH, and an aliquot dried and counted. This procedure gave low and reproducible blanks.

b. RNA pC*pC*pA* was prepared by incorporation of AMP-C^{14} and CMP-C^{14} into a preparation of venom diesterase-digested RNA (CMP:AMP = 1.87). The reaction mixture (0.25 ml) for the pyrophosphorolysis of the RNA pC*pC*pA* contained 10 μmoles of Tris buffer (pH 7.5), 2.5 μmoles of 2-mercaptoethanol, 1.5 μmoles of $MgCl_2$, 2.5 μmoles of KF, 1.75 μmoles of PP_i, 0.16 μmole of RNA nucleotide containing 2230 c.p.m., and 0.014 unit of enzyme. After incubation, 0.8 mg of serum albumin and 2 ml of 0.35 N perchloric acid were added. The precipitate was centrifuged, washed once with 2 ml of perchloric acid, dissolved in 1 ml of 1.5 M NH_4OH, and an aliquot counted.

TABLE VI

Distribution of CMP-C^{14} and AMP-C^{14} in terminal and nonterminal positions of RNA

The isolated C^{14}-labeled RNA was hydrolyzed with either 0.3 N NaOH or 1 N NaOH at 37° for about 16 hours. The nucleoside fraction and the nucleotide fractions were separated on Dowex 1-formate columns (17). The predicted values are based on a sequence of . . . pCpCpA and are calculated from the ratio of CMP to AMP incorporation obtained with the particular RNA preparation. For example, in Line 4 the calculation is obtained by considering that 0.87 mμmole of CMP is incorporated internally whereas 1.0 is terminal; therefore the percentage of terminal CMP is (1.0/1.87) 100, or 54%.

C¹⁴-labeled RNA degraded with alkali	Ratio of CMP to AMP incorporation	Position of incorporated nucleotide		Amount of nucleotide in terminal position	
		Terminal	Non-terminal	Found	Predicted
		mμmoles		*%*	
AMP-C¹⁴-RNA..................	0.85	5.6	0.34	94	100
CMP-C¹⁴-RNA..................	1.08	3.04	0.47	87	93
AMP-C¹²-CMP-C¹⁴-RNA.......	1.08	0	3.49	0	0
CMP-C¹⁴-RNA..................	1.87	4.02	2.78	59	54
AMP-C¹²-CMP-C¹⁴-RNA.......	1.87	0	6.07	0	0

Regeneration of Amino Acid-Acceptor Activity after Incorporation of AMP and CMP into Diesterase-digested or Pyrophosphorolyzed RNA—As previously reported (3), venom diesterase digestion of amino acid-acceptor RNA, under conditions in which
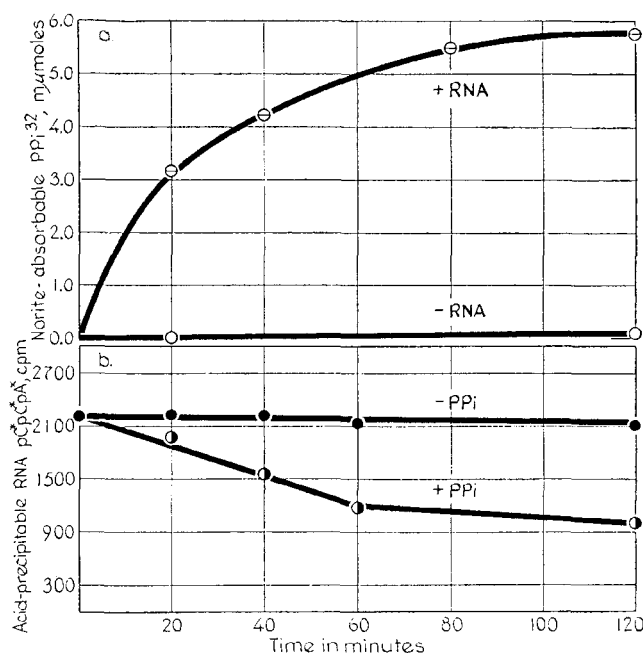
one or only a few nucleotides were cleaved from the 3'-hydroxyl end of the chains, resulted in inactivation of the amino acid acceptor activity. Pyrophosphorolysis of the RNA under the conditions described above likewise led to a loss of about 80 to 85% of the ability to accept leucine and valine (Table VIII). Incorporation of AMP and CMP into such inactivated preparation restored essentially all the amino acid acceptor activity as judged by the formation of leucyl- and valyl RNA. This reactivation was completely dependent in each case upon the incorporation of AMP, but only partially upon the incorporation of CMP. This finding of only a partial requirement for CMP addition in the restoration of amino acid acceptor activity can be attributed to the types of RNA chains remaining after venom diesterase digestion or pyrophosphorolysis. Because the dies-

TABLE VII

Identification of CTP and ATP as products of pyrophosphorolysis of amino acid-acceptor RNA

Pyrophosphorolysis was carried out with essentially the same reaction mixture described for Fig. 6a. Enzyme, 0.024 unit, PP_i^{32}, 5 μmoles, (2.8 × 10⁶ c.p.m. per μmole), and 0.92 μmole of intact amino acid-acceptor RNA nucleotide or 1.8 μmoles of venom diesterase-digested RNA nucleotide were incubated for 2 hours at 37°. The mixtures were heated for 1 minute at 100° and 1 μmole each of ATP, GTP, UTP, CTP, and ADP were added. The nucleotides were adsorbed to Norit, and then they were eluted with 50% ethanol-0.3 M NH₄OH. Between 52 and 60% of the ultraviolet-absorbing material was eluted, and after removal of the alcohol the nucleotides were adsorbed to Dowex 1-Cl⁻. Nucleoside diphosphates were eluted as one peak with 0.05 M LiCl-0.01 N HCl and these contained less than 1% of the radioactivity applied to the column. CTP was eluted with 0.08 M LiCl-0.01 N HCl; ATP, followed by a combined peak of GTP and UTP was eluted with 0.2 M LiCl-0.01 N HCl. In both experiments, the recovery of radioactivity from the column was 90 to 97%. In each analysis, the radioactivity eluted from the column coincided with the ultraviolet-absorbing peaks corresponding to ATP and CTP. These components were identified by their absorption spectra and by the order of their elution. For purposes of calculation of the yield of ATP^{32} and CTP^{32} formed in the pyrophosphorolysis reaction, correction was made for the approximately 55% recovery from the Norit.

RNA pyrophosphorolyzed	PP_i^{32} incorporated into		
	CTP	ATP	GTP + UTP
	mμmoles/μmole RNA nucleotide		
Intact amino acid-acceptor RNA	6.9	7.2	<0.1
Diesterase-digested amino acid-acceptor RNA	2.8	0.6	<0.1

terase attacks RNA chains at random (27, 35, 36), one would expect, after a short period of digestion, to obtain a population of RNA chains in which one (AMP), two (AMP and one CMP), three (AMP and two CMP residues), or more than three nucleotides have been removed. Those RNA chains with only AMP removed would be reactivated by the incorporation of only AMP. The chains which require the addition of CMP, in addition to AMP, are those which have sustained two or three hydrolytic hits. A similar explanation, that is, random removal of nucleotide end units by pyrophosphorolysis, may explain the finding of residual intact chains, chains which have had only AMP removed and chains which have also had an AMP and one or two CMP residues pyrophosphorolyzed. Consistent with this interpretation is the finding that whereas the ratio of CMP to AMP which can be incorporated after the first pyrophosphorolysis was 1.2, a second and third treatment of the isolated RNA and PP_i and the enzyme yield preparations in which CMP and AMP were incorporated in the ratio of 1.81 and 2.04.

DISCUSSION

Earlier studies (3, 10, 11) established that in the formation of amino acyl RNA derivatives each amino acid is linked to a different and specific RNA chain by an ester bond to the 2'- or 3'-hydroxyl group of a terminal adenylic acid residue. The

TABLE VIII

Regeneration of amino acid acceptor activity of pyrophosphorolyzed and diesterase-digested RNA by addition of AMP and CMP

The venom diesterase-treated RNA was prepared by incubating amino acid-acceptor RNA with purified venom diesterase in a ratio of 18.6 units of enzyme per μmole of RNA nucleotide for 15 minutes at 37° in 0.05 M glycine buffer, pH 9.2, containing 0.01 M MgCl₂. The reaction was terminated by adjusting the pH to 7.0 and heating for 1.5 minutes at 100°. The RNA was isolated by precipitation with alcohol and then dialyzed against several changes of 0.2 M NaCl followed by dialysis against water. The pyrophosphorolyzed RNA was prepared as follows: 17.5 ml contained 70 μmoles of Tris buffer (pH 7.5), 105 μmoles of MgCl₂, 17.5 μmoles of 2-mercaptoethanol, 175 μmoles of KF, 88 μmoles of PP_i, 31 μmoles of RNA nucleotide, and 0.17 mg of enzyme (DEAE-fraction B). After 120 minutes at 37°, the RNA was precipitated with 2 volumes of alcohol, washed once with 67% ethanol-0.5 M NaCl, and dissolved in 0.2 M NaCl. The RNA solution was dialyzed overnight against several changes of 0.2 M NaCl and then against water to remove the NaCl.

Incorporation of AMP and CMP was carried out to a limit as described in "Methods," and the RNA was then tested for its ability to accept leucine-C¹⁴ and valine-C¹⁴ with purified leucyl- and valyl RNA synthetases as previously described (1).

RNA tested for amino acid acceptor activity	Formation of amino acyl RNA	
	Leucine	Valine
	mμmoles/μmole RNA nucleotide	
Initial RNA	0.84	0.76
Above reacted with ATP, CTP, and enzyme	0.84	0.79
Diesterase-digested RNA	0.07	0.06
Above reacted with ATP, CTP, and enzyme	0.81	0.71
Above reacted with ATP and CTP	0.06	0.05
Above reacted with CTP and enzyme	0.07	0.05
Above reacted with ATP and enzyme	0.26	0.24
Pyrophosphorolyzed RNA	0.16	0.11
Above reacted with ATP, CTP, and enzyme	0.86	0.71
Above reacted with ATP and CTP	0.13	0.10
Above reacted with CTP and enzyme	0.13	0.10
Above reacted with ATP and enzyme	0.33	0.25

present experiments show that in the acceptor RNA of *E. coli*, as was reported for the analogous RNA from mammalian cells (14, 15), these terminal adenylic acid groups form part of a terminal trinucleotide sequence, adenylyl 5' → 3' cytidylyl 5' → 3' cytidylyl 5' → 3'-RNA. It seems certain, therefore, that the compositional or structural features which differentiate one amino acid-specific chain from another reside in another portion of the RNA molecule. Recent studies by Zillig *et al.* (38) and by Singer and Cantoni (39) have shown that amino acid-acceptor RNA chains are terminated at the nonacceptor end by guanylic acid bearing a free 5'-phosphomonoester group. Studies are now in progress to determine whether differences in nucleotide sequence exist amongst the nucleotides distal to the third cytidylic acid residue.

Although our present studies indicate that a single enzyme catalyzes the incorporation of AMP and CMP, a definitive con-

clusion must await more extensive purification and detailed studies of the reaction mechanism. In this regard, Canallakis and Herbert (15) have isolated an enzyme which incorporates both AMP and CMP, although Hurwitz (16) has reported the purification of an enzyme from thymus which appears to be specific for the incorporation of CMP. If the enzyme we have isolated does indeed incorporate both nucleotides, then the specificity of this enzyme is quite remarkable. Not only is it inactive with GTP and UTP, or with the deoxyanalogues of ATP and CTP, but it incorporates the AMP and CMP in the very precise order of 2 CMP residues and 1 AMP. One wonders what mechanisms could limit the incorporation to 2 CMP and 1 AMP and in the order . . . pCpCpA!

One of the limitations inherent in the use of snake venom phosphodiesterase for the degradation of the amino acid-acceptor RNA is the difficulty in controlling the degree and type of cleavage which occurs. Because the enzyme attacks RNA chains at random, one would expect to obtain a mixture of chains which have had 1, 2, 3, and 4 or more hydrolytic hits. Moreover, it is difficult to eliminate entirely endonuclease activity which leads to cleavage of internal diester linkages (35). We have obtained preliminary data which suggest that chains which have more than the terminal trinucleotide removed (*i.e.* those chains which have been degraded past the fourth nucleotide or those which have had internal bonds cleaved) are inert as CMP or AMP acceptors. Thus, the total amount of CMP which can be incorporated increases and then begins to decrease as the diesterase digestion proceeds. We interpret these findings as indicating that the enzyme can add CMP only to the preexisting fourth nucleotide or to some sequence or configuration involving the fourth nucleotide. Some support for this interpretation is derived from studies which show that in the pyrophosphorolytic reaction ATP and CTP but no GTP or UTP are formed. This was true even in the case with RNA, which had most of the AMP and CMP removed by prior degradation with diesterase. This suggests that pyrophosphorolysis proceeds up to, but not past, the fourth nucleotide. It is possible however that one or more enzymes exist which, in conjunction with the AMP- and CMP-incorporating enzyme, could effect a more complete pyrophosphorolysis and resynthesis of the RNA. In this connection one should recall the finding by Harbers and Heidelberger (17) of GMP and UMP incorporation into RNA. It would be of considerable importance to learn whether the AMP- and CMP-incorporating enzyme is one of several similar enzymes involved in the complete synthesis of amino acid-acceptor RNA chains or whether it serves to regenerate terminal groupings of the RNA as it functions in protein synthesis (39).

SUMMARY

Amino acid-acceptor ribonucleic acid from *Escherichia coli*, after partial digestion with snake venom phosphodiesterase, serves as acceptor for adenosine phosphate and cytosine phosphate. These nucleotides are incorporated from their respective triphosphates by an enzyme purified from *E. coli*. Inorganic pyrophosphate is formed in amounts equivalent to the amount of nucleotide incorporated into the ribonucleic acid. The reaction is reversible; pyrophosphorolysis of intact amino acid-acceptor ribonucleic acid leads to a net formation of only adenosine triphosphate and cytosine triphosphate. Depending upon the extent of prior digestion of the ribonucleic acid by the diesterase, either adenosine phosphate, or adenosine phosphate and 1 cytosine phosphate or adenosine phosphate and 2 cytosine phosphate residues are incorporated. Alkaline degradation studies show that the two cytosine phosphate residues are adjacent to each other and that adenosine phosphate is linked to the terminal cytosine phosphate. These studies establish that the terminal trinucleotide sequence of the amino acid-acceptor ribonucleic acid of *E. coli* is adenylyl 5′ → 3′ cytidylyl 5′ → 3′ cytidylyl 5′ → 3′ ribonucleic acid.

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